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Japan

Food and Agricultural Import Regulations and Standards

Designation of Nisin as a Food Additive

2008

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Report Highlights:
On March 24, 2008, MHWL proposed Designation of Nisin as a Food Additive.

Includes PSD Changes: No
Includes Trade Matrix: No
Annual Report
Tokyo [JA1]
[JA]

Executive Summary
On March 24, 2008, MHWL proposed Designation of Nisin as a Food Additive.

Purpose
The period for comments directly to MHLW closed already on April 7, 2008. However MHLW will also notify these proposed changes to the WTO/SPS committee, which would be the last chance for public comments to be submitted on this subject. Then after the closing of a the comment period in the WTO, a report to the Minister of Health, Labour, and Welfare will be made based on the conclusions of a session of the Pharmaceutical Affairs and Food Sanitation Council slated to be held at a later date, and this will constitute the final decision.

If you have comments that you would like to be considered for inclusion in the official U.S. Government comments to the MHLW, please send those as soon as possible to the Agriculture Section of the U.S. Embassy in Tokyo, at agtokyo@usda.gov.

For comments directly to MHLW, please send those to following contacts.

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Outline

Under Article 10 of the Food Sanitation Law, food additives can be used or marketed only when they are designated by the Minister of Health, Labour and Welfare. When use standards or compositional specifications are established for food additives, based on Article 11 of the law, those additives are not permitted to be marketed unless they meet these standards or specifications.

In response to a request from the Minister, the Subcommittee on Food Additives under the Food Sanitation Committee under the Pharmaceutical Affairs and Food Sanitation Council has discussed the adequacy of the designation of Nisin. The subcommittee has concluded as follows.

Conclusion from the subcommittee

The Minister should designate Nisin, based on Article 10 of the Food Sanitation Law, as a food additive unlikely to harm human health and establish compositional specifications for the substance, based on Article 11 of the law (see Attachment 2-1).

Additional Information

A list showing progress in the designation procedure of food additives that have been proven safe by JECFA (Joint FAO/WHO Expert Committee on Food Additives) and that are widely used in countries other than Japan (Attachment 2-2)

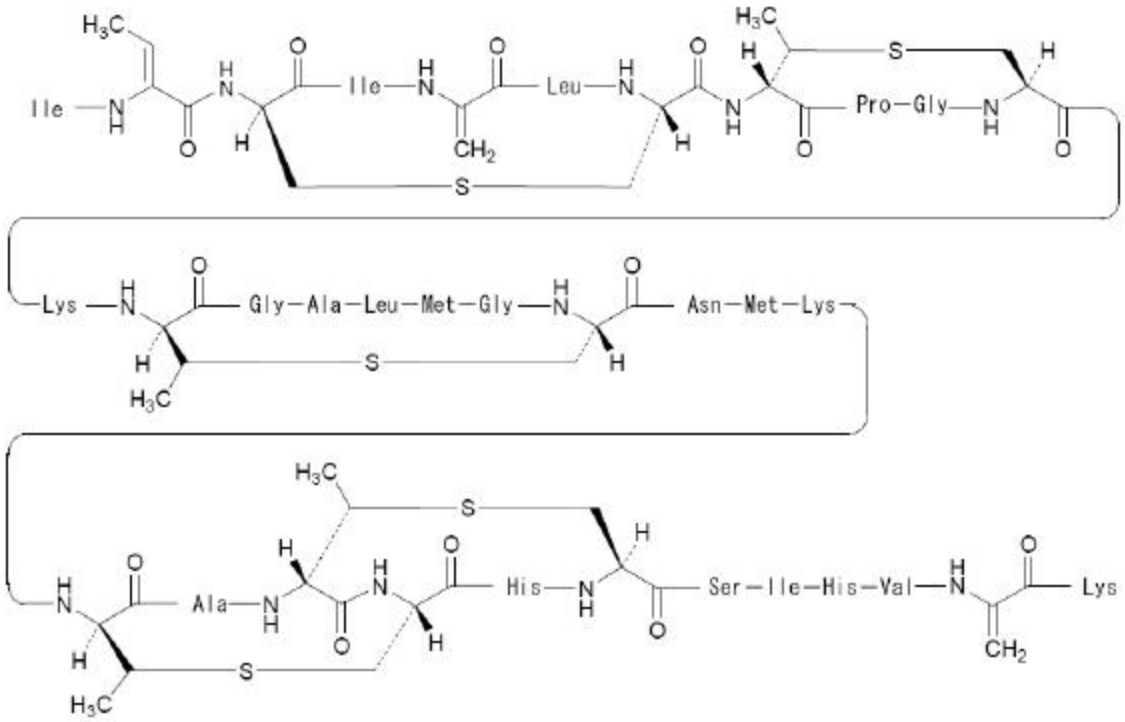
1. **Standards for use**

See the table.

2. **Compositional specifications**

Substance name Nisin

Structural formula



Molecular formula $C_{143}H_{230}N_{42}O_{37}S_7$

Mol. Weight 3354.07

CAS number [1414-45-5]

Definition A mixture of sodium chloride and antimicrobial polypeptides obtained from a culture fluid of *Lactococcus lactis* subsp. *lactis*. It contains components derived from nonfat milk medium or sugar medium. The main antimicrobial polypeptide is nisin A.

Potency and content Nisin contains not less than 900 units of potency per milligram of Nisin. The potency is the amount of nisin ($C_{143}H_{230}N_{42}O_{37}S_7$), expressed with the number of units. One unit is equivalent to 0.025 μg of nisin ($C_{143}H_{230}N_{42}O_{37}S_7$). The

product contains not less than 50% of sodium chloride.

Description A white to pale yellowish white powder. It is odorless or has a slight, characteristic odor.

Identification (1) Suspend 0.100 g of Nisin, exactly weighed, in 80 ml of 0.02 mol/L hydrochloric acid, previously sterilized by passing through a 0.2 μm filter. Allow to stand for 2 hours at room temperature, and add sterilized 0.02 mol/L hydrochloric acid to make 100 ml. Dilute 1 ml of the resulting solution, exactly measured, to exactly 200 ml with sterilized 0.02 mol/L hydrochloric acid, and use it as the control solution. Prepare a test solution by boiling 20 ml of the control solution for 5 minutes. Determine the potency of both solutions as directed under the Assay. The potency of the test solution is in the range of 100%±5% of that of the control solution.

Separately, adjust 20 ml of the test solution to pH 11 by adding 5 mol/L sodium hydroxide, and heat at 65°C for 30 minutes. After cooling, adjust the pH to 2.0 by adding hydrochloric acid, and again determine the potency as directed under the Assay. Complete loss of the antimicrobial activity of Nisin is observed.

(2) Prepare a test culture fluid by incubating *Lactococcus lactis* (ATCC 11454 or NCIMB 8586) in a 1 in 10 solution of sterile powdered skim milk at 30°C for 18 hours. Sterilize a flask containing 100 ml of litmus milk in an autoclave at 121°C for 15 minutes, add 0.1 g of Nisin, and allow to stand for 2 hours at room temperature. To the resulting solution, add 0.1 ml of the test culture fluid, and incubate at 30°C for 24 hours. The growth of *Lactococcus lactis* is observed.

Purity (1) Lead Not more than 1.0 μg/g as Pb.

Sample Solution Weigh 10.0 g of Nisin into a heat-resistant beaker containing 5 ml of sulfuric acid, heat gradually, and add a small amount of sulfuric acid. Incinerate at the lowest possible temperature, ignite at 500°C until it is completely incinerated, and cool. Dissolve the residue by adding 40 ml of water.

Test Solution To the sample solution, add 10 ml of a solution of diammonium citrate (1 in 2), and make it weakly alkaline with aqueous ammonia using thymol blue TS as the indicator. After cooling, transfer this solution into a 200-ml separating funnel, wash the beaker with water, and add the washings to the separating funnel, and make about 100 ml. Add 5 ml of a solution of ammonium pyrrolidine dithiocarbamate (3 in 100), allow to stand for 5 minutes, and add 10 ml of butyl acetate. Shake for 5 minutes, and allow to stand. Use the butyl acetate layer as the test solution.

Control Solution Add water to 1 ml of the Lead Standard Stock Solution, exactly measured, to make exactly 100 ml. Using 10 ml of the resulting solution, exactly measured, proceed as directed under *Test Solution*.

Procedure Determine as directed in Method 1 under the Lead Limit Test.

(2) Arsenic Not more than 2.0 μg/g as As_2O_3 (1.0 g, Method 3, Apparatus B).

Loss on Drying Not more than 3.0% (105°C, 2 hours).

Microbial Limit Tests Proceed as directed under the Microbial Limit Tests. The total bacteria count is not more than 100/g of Nisin, and *Escerichia coli* is negative. *Salmonella* is negative when the test is performed as directed below.

Bacteria Count Use the Membrane Filter Method. Prepare a sample solution by mixing 1 g of Nisin with sodium chloride-peptone buffer to make a 1000 ml solution. Filter 100 ml of the sample solution through a cellulose ester membrane filter, and wash the filter with sodium chloride–peptone buffer (other solutions specified in the Membrane Filter Method may be used as well). Place the filter on soybean-casein digest agar medium, and incubate for 5 days at 30–35°C.

Escerichia coli Prepare a sample solution by adding fluid lactose broth medium to 1 g of Nisin to make 100 ml, and incubate it for 24–72 hours at 30–35°C.

Salmonella Test

Procedure Add fluid lactose broth medium to 10 g of Nisin to make 200 ml, and incubate at 30–35°C for 24–72 hours. If microbial growth is observed in the culture fluid, shake the culture fluid lightly, and inoculate 1 ml-portions into fluid tetrathionate medium and fluid rappaport medium, respectively. Incubate them at 30–35°C for 18–24 hours. Inoculate appropriate portions from both fluid media onto each of brilliant green agar medium and XLD agar medium, and incubate at 30–35°C for 42–48 hours. *Salmonella* cultures typically produce small, transparent, colorless colonies or opaque, white to pink colonies on brilliant green medium, or red colonies on XLD agar medium. Colonies on brilliant green agar medium are frequently surrounded by a pink to red zone, and colonies on XLD agar medium have dark centers. If typical colonies are not present, *salmonella* is determined to be absent. If colonies of Gram-negative rods with the typical characteristics are observed, inoculate suspect colonies to TSI slant agar medium, using both slant and deep inoculation techniques, by means of an inoculating needle, and incubate for 42–48 hours at 30–35°C. In the presence of *salmonella*, the color changes from red to yellow in the deep culture, and the slant surface remains unchanged (red). Usually, there is a formation of gas with or without the production of hydrogen sulfide in the medium. It is desirable to conduct biochemical and serological tests, including use of an identification kit, for definitive identification and serotyping.

Effectiveness of culture media and confirmation of anti-microbial substances

For the test, use nonpathogenic or weakly pathogenic strains of *salmonella*, incubated in fluid lactose broth medium at 30–35°C for 18–24 hours. Prepare suspensions with each containing 1000 viable microorganisms per ml, using sodium chloride–peptone buffer solution, phosphate buffer, or fluid lactose broth medium. If necessary, by adding 0.1-ml portions of a *salmonella* suspension (with each containing 1,000 viable microorganisms/ml), examine the effectiveness of the media to be used and

the presence of anti-microbial substances, both in the presence and in the absence of the sample.

Confirmation

If uncertain or doubtful results are obtained, validate the test method with an amount of sample 2.5 times that used in the original test, as directed under *Procedure*. Adjust the amounts of medium and reagents used in proportion to an increase of the sample.

Media

(i) Fluid tetrathionate medium

Casein peptone	2.5 g
Meat peptone	2.5 g
Sodium desoxycholate	1.0 g
Calcium carbonate	10.0 g
Sodium thiosulfate pentahydrate	30.0 g
Water	1,000 ml

Mix the ingredients, and dissolve by boiling. On day of use, add a solution prepared by dissolving 5g of potassium iodide and 6 g of iodine in 20 ml of water. Then add 10 ml of sterilized brilliant green solution (1 in 1,000), and mix well. Thereafter, do not heat the midium.

(ii) Fluid rappaport medium

Soybean peptone	5.0 g
Sodium chloride	8.0 g
Potassium dihydrogen phosphate	1.6 g
Malachite green oxalate	0.12 g
Magnesium chloride hexahydrate	40.0 g
Water	1,000 ml

Separately dissolve malachite green oxalate, magnesium chloride hexahydrate, and the remaining three solid ingredients in water, and separately autoclave them at 121°C for 15–20 minutes, and mix. The pH is 5.4–5.8.

(iii) Brilliant green agar medium

Peptone (meat or casein)	10.0 g
Yeast extract	3.0 g
Sodium chloride	5.0 g
Lactose monohydrate	10.0 g
White sugar	10.0 g
Phenol red	0.080 g
Brilliant green	0.0125 g
Agar	20.0 g

Water	1,000 ml

Mix the ingredients, and boil for 1 minute. Immediately before use, autoclave at 121°C for 15–20 minutes. The pH is 6.7–7.1 after sterilization. Cool to about 50°C, and dispense to Petri dishes.

(iv) XLD (xylose·lysine·desoxycholate) agar medium

Xylose	3.5 g
L-Lysine monohydrochloride	5.0 g
Lactose monohydrate	7.5 g
White sugar	7.5 g
Sodium chloride	5.0 g
Yeast extract	3.0 g
Phenol red	0.080 g
Sodium desoxycholate	2.5 g
Sodium thisulfate pentahydrate	6.8 g
Ammonium iron (III) citrate	0.80 g
Agar	13.5 g
Water	1,000 ml

Mix the ingredients, and dissolve by boiling. Avoid excessive heating. The pH is 7.2–7.6 after boiling. Do not autoclave. Cool to about 50°C, and dispense to Petri dishes.

(v) TSI (triple sugar iron) agar medium

Casein peptone	10.0 g
Meat peptone	10.0 g
Lactose monohydrate	10.0 g
White sugar	10.0 g
Glucose	1.0 g
Ammonium iron (II) sulfate hexahydrate	0.20 g
Sodium chloride	5.0 g
Sodium thisulfate pentahydrate	0.20 g
Phenol red	0.025 g
Agar	13.0 g
Water	1,000 ml

Mix the ingredients, and dissolve by boiling. Dispense into small test tubes. Autoclave at 121°C for 15–20 minutes. The pH is 7.1–7.5 after sterilization. Use as slant agar medium. The medium containing 3 g of meat extract or years extract additionally or the medium containing ammonium iron (III) citrate instead of Ammonium iron (II) sulfate hexahydrate may be used.

Assay (1) Potency

Antimicrobial activity (potency) is determined by use of perforated agar plates, based on the size of zone of growth inhibition of a test organism. As necessary, sterilize water, regents, solutions, and other equipment used for the test.

(i) *Assay Organism* Use *Micrococcus luteus* (ATCC 10240, NCIMB 8166)

(ii) *Media* Adjust the pH of each medium, using sodium hydroxide TS or 1 mol/L hydrochloric acid, so that it is the specified value after sterilization. Use the high-pressure steam method for sterilization. Media other than those given below may be used as well if they consist of similar ingredients to the specified media and they are comparable or superior in efficiency of microbial growth to the specified media.

a. Agar medium for inoculated medium

Tryptone	10 g
Beef extract	3 g
Sodium chloride	3 g
Yeast extract	1.5 g
Sucrose	1 g
Agar	15 g
Water	1,000 ml

Mix the ingredients, and sterilize at 121°C for 15 minutes. The pH is 7.4–7.6 after sterilization. Add 2 ml of 50% polysorbate 20 solution of the same temperature as the medium.

b. Agar medium for assay organism incubation

Brain heart infusion agar	52 g
Water	1,000 ml

Mix the ingredients, and sterilize at 121°C for 15 minutes. The pH is 7.2–7.6 after sterilization. Dispense 9-ml potions into test tubes (16 mm internal diameter) and prepare slant media.

(iii) *Assay Culture Fluid* Using agar medium b above, incubate the assay organism at 30°C for 48 hours to prepare a slant culture. Suspend the growth on the slant culture in 7 ml of sterilized saline solution to prepare the assay culture fluid. The prepared slant culture may be stored for a maximum of 14 days at 4°C.

(iv) *Inoculated Agar Medium* To 100 ml of the agar medium for inoculated medium, held at 48–51°C, add 2 ml of a solution prepared by diluting the assay culture fluid with saline solution (1 in 10), and mix well.

(v) *Perforated Agar Plate* Dispense about 20-ml portions of the inoculated agar medium into Petri dishes (90 mm inter diameter, 20 mm height). If large dishes are

used, dispense the medium to form a uniform layer of 2 to 3 mm. Spread the medium evenly over the surface, and harden at room temperature. Use them as the inoculated agar plates. Place 4 stainless-steel cylinders (penicillin cups: 7.9–8.1 mm external diameter, 5.9–6.1 mm external diameter, 9.9–10.1 mm height) on about 25- to 28-mm centers on each agar plate so that the inter-central distance between cups is 30 mm or more. Pour 20 ml of the inoculated agar medium on the plate with the cylinders, and harden, and set aside at 4°C for 30–60 minutes. Remove the cylinders gently by means of appropriate sterilized equipment, such as tweezers. Use cylinders which do not interfere with the test.

(vi) *Nisin Standard Solutions* Suspend 0.100 g of Nisin Reference Standard, exactly weighed, in 80 ml of 0.02 mol/L hydrochloric acid, sterilized by passing through a 0.2-μm filter, and set aside at room temperature for 2 hours. Add sterilized 0.02 mol/L hydrochloric acid to make a 100-ml solution. Use the resulting solution as the standards stock solution (1000 IU/ml). Before use, dilute appropriate volumes of this solution with sterilized 0.02 mol/L hydrochloric acid to obtain 5 nisin standard solutions with concentrations of 1.25, 2.5, 5.0, 10.0, and 20.0 (IU/ml).

(vii) *Standard Curve* Use 5 perforated agar plates for one assay set. When large dishes are used, the total number of holes for one assay set should be equal to that defined for Petri dishes. Transfer, in quadruplicate, 0.2-ml portions from each standard solution into holes, one concentration to a plate. Cover the plates, and incubate them at 30°C for 18 hours. Measure the zone diameters of inhibition to the nearest 0.1 mm by means of a caliper or other appropriate device. Plot the log of nisin concentration (x IU/ml) on the abscissa axis and the zone diameters (y mm) on the ordinate axis to prepare the standard curve ($y = \alpha \log x + \beta$). Obtain the constants α and β.

(viii) *Determination of Nisin Concentration* Suspend 0.100 g of Nisin, exactly weighed, in 80 ml of 0.02 mol/L hydrochloric acid, sterilized by passing through a 0.2-μm filter, and set aside at room temperature for 2 hours. Add sterilized 0.02 mol/L hydrochloric acid to make 100 ml. To 1 ml of this solution, exactly measured, add sterilized 0.02 mol/L hydrochloric acid to make 200 ml. Use this solution as the test solution. Measure the zone diameters of inhibition as directed in the preparation of the calibration curve. From the calibration curve, determine the potency (IU/mg).

The test solution should be prepared fresh before use. The measurement of zones for standard solutions and test solution should be done at one time.

(ix) *Calculation of Potency* Obtain the potency of the test solution from the calibration curve.

Potency of the test solution (IU/ml) = 10^I

$$I = \frac{\text{Zone diameter (mm)} - \beta}{\alpha}$$

$$\text{Potency of sample (IU/mg)} = \frac{\text{Potency of the test solution}}{5} \times 1{,}000$$

(2) Sodium Chloride

Dissolve 0.1 g, accurately weighed, by adding 100 ml of water, and make acidic by adding nitric acid. Titrate with 0.1 mol/L silver nitrate using a silver electrode as the indicator electrode and a silver–silver chloride electrode as the reference electrode. Separately, perform a blank test to determine the volume of the 0.1 mol/L silver nitrate consumed. Obtain the sodium chloride content in the sample by the following formula.

$$\text{Content (\%) of sodium chloride (NaCl)} = \frac{a \times 5.85}{\text{Weight (g) of the sample} \times 10}$$

<Reagents and Test Solutions>

Brain Heart Infusion Agar Use agar produced for the microbial limit tests.

Diammonium Citrate $C_6H_{14}N_2O_7$ [K8284]

Litmus [K8940: 1961] Blue to purplish blue powder or lumps. Soluble in water and in ethanol. Its solution is blue to purplish blue.

Identification Dissolve 0.5 g of Litmus in warm water, add dropwise dilute sulfuric acid until the solution produces a red color, and boil for 10 minutes. If the color changes to blue during boiling, add dilute sulfuric acid. Then add a saturated solution of barium hydroxide until it produces a purple color, and filter. Refer to the resulting solution as solution A. To 100 ml of freshly boiled and cooled water, add 0.5 ml of solution A and 0.05 ml of 0.1 mol/L hydrochloric acid. A red color is produced. To 100 ml of freshly boiled and cooled water, add 0.5 ml of solution A and 0.05 ml of 0.1 mol/L sodium hydroxide. A blue color is produced.

Litomus Milk Dissolve 100 g of powdered skim milk, 0.5 g litmus, 0.5 g of anhydrous sodium sulfite by adding 100 ml of water. Sterilize the mixture at 115°C for 15 minutes.

Malachite Green Oxalate $C_{52}H_{54}N_4O_{12}$ [Malachite Green (Oxalate), K8878]

Monopotassium Phosphate See Potassium Dihydrogen Phosphate

Nisin Reference Standard Use a reference standard produced by producers registered with the Minister of Health, Labour and Welfare.

50% Polysorbate 20 Solution Mix equal volumes of polysorbate 20 and water, and autoclave at 121°C for 15 minutes.

Potassium Dihydrogen Phosphate KH_2PO_4 [K9007]

Saline Solution Use saline solution specified in the Japanese Pharmacopoeia.

Tryptone Use products produced for the microbial limit tests.

Trisodium Phosphate Dodecahydrate $Na_3PO_4.12H_2O$ [K9012]

Standards for use of Nisin

Products	Maximum use level [1] as purified nisin
Cheeses, except processed cheeses	0.0125 g/kg
Meat products	
Whipped creams	
Dressings	0.010 g/kg
Mayonnaise	
Sauces[2]	
Fine bakery products	0.00625 g/kg
Processed cheeses	
Miso	0.0050 g/kg
Processed egg products	
Moist, unbaked, sweet cakes made mainly of creal grains or starch[3]	0.0030 g/kg

Note

1. The above maximum use levels are not applied to products permmited or recognized by the Minister of Health, Labour and Welfare as foods for special dietary uses. The foods include five types of products: foods for the ill, milk powder for pregnant and lactating women, formulated milk powder for infants, foods for the aged, foods for specified health uses.
2. Sauces refer to all kinds of sauces including Oriental thick Worcester sauce, cheese souce, and ketchup, but excluding fruit sauce and its analogues used for cakes.
3. They refer to rice pudding and tapioca pudding, and their analogues, but excluding Oriental sweet dumplings.